Protection Against Myocardial Infarction and No-Reflow Through Preservation of Vascular Integrity by Angiopoietin-like 4

Running title: Galaup et al.; Protecting Vascular Integrity in Heart Ischemia

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Abstract:

**Background** - Increased permeability, predominantly controlled by endothelial junction stability, is an early event in the deterioration of vascular integrity in ischemic disorders. Hemorrhage, edema and inflammation are the main features of reperfusion injuries, as observed in acute myocardial infarction (AMI). Thus, preservation of vascular integrity is fundamental in ischemic heart disease. Angiopoietins are pivotal modulators of cell–cell junctions and vascular integrity. We hypothesized that hypoxic induction of Angiopoietin-like protein 4 (ANGPTL4) might modulate vascular damage, infarct size and no-reflow during AMI.

**Methods and Results** - We showed that vascular permeability, hemorrhage, edema, inflammation and infarct severity were increased in angptl4-deficient mice. We determined that decrease in VEGFR2 and VE-Cadherin expression and increase in Src kinase phosphorylation downstream of VEGFR2 were accentuated after ischemia-reperfusion in the coronary microcirculation of angptl4-deficient mice. Both events led to altered VEGFR2/VE-Cadherin complexes and to disrupted adherens junctions in the endothelial cells of angptl4-deficient mice that correlated with increased no-reflow. *In vivo* injection of recombinant human ANGPTL4 protected VEGF-driven dissociation of the VEGFR2/VE-Cadherin complex, reduced myocardial infarct size, and the extent of no-reflow in mice and rabbits.

**Conclusions** - These data showed that ANGPTL4 might constitute a relevant target for therapeutic vasculoprotection aimed at counteracting the effects of VEGF, thus being crucial for preventing no-reflow and conferring secondary cardioprotection during AMI.

**Key words:** No-reflow; acute myocardial infarction; endothelial cells, vascular integrity, hypoxia
Introduction

Rapid restoration of adequate myocardial reperfusion in obstructed infarct arteries is a key determinant of short- and long-term outcomes for patients with AMI. Restoration of blood supply in cardiac ischemic tissue provides oxygen and nutrients to the starved myocardium and thus limits the extent of AMI, but it also induces microvascular dysfunction, inflammation and oxidative damage. Increased vascular permeability is an important contributor to myocardial damage after ischemic events. Vascular damage also contributes to inadequate myocardial perfusion (the "no-reflow" phenomenon), which is observed in 30% of patients and is associated with a worse prognosis at follow-up and higher incidence of death. Prevention of vascular leak and edema improves flow during reperfusion post-AMI. Enhancing myocardial flow therefore represents a pertinent strategy to improve ongoing therapies that might limit infarct size, ameliorate cardiac function, and prolong survival. Treatments that minimize microvascular damage should be prioritized to protect the injured myocardium as recommended by guidelines set by the American College of Cardiology/American Heart Association (ACC/AHA). However, pharmacological treatment that targets microvascular dysfunction and inhibits the no-reflow phenomenon after reperfusion is not available.

Angiopoietins are major regulators of angiogenesis and vascular leakage through binding to the Tie2 receptor. Angiopoietin-1 promotes vessel maturation whereas Angiopoietin-2 destabilizes interactions between the cells of the vascular wall responsible for endothelial barrier disruption and vascular leakage. Angiopoietin-like proteins (ANGPTL1-7) share structural and functional properties with Angiopoietins but do not bind to the Tie2 receptor. Angiopoietin-like protein 4 (ANGPTL4) is a secreted 55-kDa protein that is processed in 20-kDa and 35-kDa forms. It is dual-function protein: an inhibitor of lipoprotein and hepatic lipases.
with angiogenic properties. We have shown that its expression is induced by hypoxia in vascular cells in ischemic diseases. ANGPTL4 is a tissue-specific modulator of vascular permeability that regulates the survival and adhesion of endothelial cells (ECs) in vitro and angiogenesis in vivo. In humans, ANGPTL4 possesses several variants, including the inactive E40K variant, which is associated with significantly lower plasma levels of triglycerides (TG) and higher levels of high-density lipoproteins (HDLs). Interestingly, individuals carrying the E40K variant display an atheroprotective lipid profile but are subjected to an increased risk of coronary heart disease.

We show here that ANGPTL4 expression is induced in response to myocardial infarction in humans and mice. The goal of the present study was to analyze the role that ANGPTL4 might play in this context. Indeed, ANGPTL4 is expressed by cardiomyocytes, but also in ECs in response to hypoxia, and could modulate vascular permeability. In a model of myocardial ischemia-reperfusion, we show that angptl4-deficient mice display: (i) increased size of myocardial infarcts, (ii) increased no-reflow, (iii) decreased vascular integrity through Src-dependent dissociation of the VEGFR2/VE-Cadherin complex and subsequent destabilization of endothelial adherens junctions, and (iv) increased hemorrhage and inflammation. In addition, we show that recombinant human ANGPTL4 (rhANGPTL4) has therapeutic potential in reducing no-reflow, hemorrhage and infarct size after myocardial infarction in mice and in a non-rodent species (rabbit). These data therefore provide evidence that vasculoprotection through ANGPTL4 is a relevant therapeutic approach to confer secondary cardioprotection in AMI.

Material and Methods
The experiments were performed in accordance with the guidelines of the French Ministry of Agriculture. This study conforms to the standards of INSERM (the French National Institute of Health) in accordance with European Union Council Directives (86/609/EEC). All experiments were performed blindly, meaning that the experimenter was blind to the mouse genotype.

Animals and genotyping: \( \text{Angptl4}^{\text{LacZ/LacZ}} \) mice in which the \( \text{angptl4} \) locus was replaced by a \( \text{lacZ} \) reporter gene were generated and genotype was determined by PCR of tail genomic DNA. Eight to 12 weeks of age \( \text{angptl4}^{\text{LacZ/}+} \) and \( \text{angptl4}^{\text{LacZ/LacZ}} \) knock-out male mice, intercrossed in C57/Bl6 mice for more than 8 generations, were subjected to myocardial infarction protocols or used as control in basal conditions.

Myocardial ischemia-reperfusion experiments: Ischemia-reperfusion protocol was performed on \( \text{angptl4}^{\text{LacZ/}+} \) and \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice or rabbits using a standard technique described in Supplemental Materials. Mice and rabbits randomly received either vehicle or human recombinant 55 kDa full-length ANGPTL4 (rhANGPTL4 10 \( \mu \)g/kg i.v.).

Modified Miles assay: Male \( \text{angptl4}^{\text{LacZ/LacZ}} \) and \( \text{angptl4}^{\text{LacZ/}+} \) mice were anesthetized using pentobarbital. Under basal conditions, mice were injected into the tail vein with 1% Evans blue (200 \( \mu \)L) and sacrificed 4 h later. After ischemia-reperfusion, mice were subjected to coronary occlusion for 45 min and intravenously injected with 1% Evans blue (200 \( \mu \)L) prior the 4 h of reperfusion. At sacrifice, mice were perfused through the aorta with citrate buffer pH4. Blood, dye and buffer exited through an opening in the right atrium. Evans Blue was eluted for 18 h at 70°C in 1mL formamide. After centrifugation, absorbance at 620 nm was measured using a
spectrophotometer. Extravasated Evans blue (ng) was determined from a standard curve and normalized to tissue weight (g).

**Immunofluorescence study and confocal analysis on cryosections:** Immunofluorescence staining was performed as previously described²⁸ and confocal analysis on cryosections is detailed in Supplemental Material.

**Immunoprecipitation and immunoblotting analyses:** For *in vivo* samples, mice subjected to 45 min ischemia followed by 4 h or 18 h reperfusion, were anesthetized, injected into tail vein with 1 mM Na₃VO₄ and 2 mM H₂O₂ and dissected to remove left ventricle. For HUAECs experiments, 40000 cells/cm² were seeded in complete culture medium (Promocell) for 72 h. Cells were starved overnight and treated for 5 min with 100 ng/mL human recombinant VEGF₁₆₅ (Sigma) or with a mix containing 100 ng/mL VEGF and 5 μg/mL human recombinant ANGPTL4¹². After two washes using Ca²⁺/Mg²⁺PBS, proteins were extracted, immunoprecipitated for VEGFR2 and analyzed by Western blot as described in Supplementary Material.

**Isolation of cardiomyocytes and viability assay:** Cardiac myocytes were isolated as described in Supplemental Material and incubated in an anaerobic chamber containing a humidified atmosphere of 5% CO₂ and 95% N₂ for 3 h. Experimental medium was changed to serum-free, glucose-free. Cardiac myocyte survival was measured by staining cells with trypan blue (Sigma).

**Statistical analyses:** All data were analysed using non parametric tests. Overall comparisons were performed either using Kruskall-Wallis test (1 factor – 3 groups ) or Friedman test (2
factors – 2 groups). Individual comparisons were examined with a Mann-Whitney test. The use of Bonferroni correction allowed the adjustment for multiple comparisons. Differences were considered significant with *, p<0.05.

Results

Early post-ischemic vascular integrity is altered in angptl4LacZ/LacZ mice

Angptl4LacZ/LacZ mice in which the angptl4 locus was replaced by a lacZ reporter gene were generated 26. We first analyzed vascular permeability after 45 min ischemia and 4 h reperfusion in the heart of angptl4LacZ/LacZ and angptl4LacZ/+ mice. Histological analyzes showed that tissue damage was equivalent in both groups at this early time point (data not shown). In contrast, angptl4LacZ/LacZ mice displayed an increased vascular leakage of Evans blue dye compared with angptl4LacZ/+ mice 4 h after ischemia (117.5±8.7 versus 67.8±17 μg/mL, p<0.05), whereas no significant difference was observed under basal conditions (Fig.1A). Importantly, angptl4LacZ/LacZ mice did not exhibit functional cardiac defects (Supplemental Table 1) or abnormal vascular morphology, as examined by CD31 and NG2 staining (which was used to label endothelial cells and pericytes, respectively) under basal (Supplemental Figures 1A and 1B) and ischemic conditions (Supplemental Figures 1C and 1D).

Then, fluorescent microspheres were injected to label sites of vascular leakage. Under basal conditions, FITC-beads did not extravasate in either group (Figures 1B, 1C, Supplemental Figures 1A and 1B). After 45 min of ischemia and 4 h of reperfusion, FITC-beads were not observed in angptl4LacZ/+ mice (Figures 1D and Supplemental Figure 1C) whereas extravasation of fluorescent microspheres was detected in angptl4LacZ/LacZ mice, indicating that endothelial integrity was altered (see asterisks in Figure 1E and Supplemental Figure 1D). The stability of adherens junctions is critical for the maintenance of the endothelial permeability and integrity.
Hence, we sought to investigate VE–Cadherin distribution at endothelial adherens junctions under basal conditions and after ischemia-reperfusion. In non-ischemic myocardium from both genotypes, VE–Cadherin linear signal labeling a dense vascular network was observed (Figure 1B and 1C). After ischemia-reperfusion, a heterogeneous pattern of VE-Cadherin staining was observed; both intense and linear signals were adjacent to thinner signals in \textit{angptl4}^{LacZ/+} mice (arrows and arrowheads, respectively, in Figures 1D and 1E). In \textit{angptl4}^{LacZ/LacZ} mice, ischemia-reperfusion injury induced more severe damage at endothelial junctions, which were mainly disrupted as shown by more systematic discontinuous VE-Cadherin staining (open arrowheads in Figure 1E). Three-dimensional reconstruction from confocal images stained with anti-VE–Cadherin (Figures 1F and 1G) or with anti-CD31 and anti-NG2 (Supplemental Figures 1E and 1F) antibodies further confirmed disrupted EC junctions and extravasated FITC-beads in \textit{angptl4}^{LacZ/LacZ} coronary vessels.

These observations suggested that coronary vascular integrity was severely disrupted and junction disassembly more frequent in \textit{angptl4}^{LacZ/LacZ} mice compared with \textit{angptl4}^{LacZ/+} mice after ischemia/reperfusion, thereby leading to increased vascular permeability.

**Post-ischemic decrease in the expression of VEGFR2 and VE-Cadherin combined to increase Src kinase phosphorylation downstream of VEGFR2 in \textit{angptl4}^{LacZ/LacZ} mice**

In the vasculature, VEGFR2 and VE-Cadherin form complexes that are transiently dissociated upon VEGF binding to VEGFR2. During myocardial infarction, ischemia promotes VEGF expression\textsuperscript{29} that leads to vascular permeability and edema\textsuperscript{30}. We therefore investigated if disassembly of the VEGFR2/VE-Cadherin complex might constitute the mechanism...
responsible for increased junctional disruption in angptl4LacZ/LacZ mice after ischemia-reperfusion injury.

Using RT–qPCR methodology, the expression of vegfr2 and ve-cadherin mRNA were quantified in the left ventricle in angptl4LacZ/+ and angptl4LacZ/LacZ mice under control conditions or after 4 h or 18 h reperfusion. As shown in Supplemental Figure 2A, vegfr2 (left) and ve-cadherin (right) mRNA expression was similar in both groups under basal conditions. After 4 h reperfusion, the decrease in vegfr2 expression was more prominent in angptl4LacZ/LacZ mice compared with control mice (66±5% versus 44±6% for vegfr2, p<0.05). Ve-cadherin mRNA expression was unchanged angptl4LacZ/LacZ mice compared with control mice. Vegfr2 mRNA down-regulation was maintained after 18 h reperfusion (decrease of 60±4% for angptl4LacZ/LacZ mice versus 10±5% for angptl4LacZ/+ mice, p<0.05). VEGFR2 and VE-Cadherin protein levels were strongly affected at 18-h reperfusion as shown by western blot analyzes undertaken using total extracts from left ventricles (Supplemental Figure 2B).

Src signaling transduces VEGF-mediated permeability by dissociating the VEGFR2/VE-Cadherin complex. To further determine the mechanism leading to early post-ischemic junction alteration in angptl4LacZ/LacZ mice, Src kinase signaling downstream of VEGFR2 was analyzed under control conditions and after ischemia-reperfusion. Left-ventricle lysates were immunoprecipitated for VEGFR2 followed by immunoblotting for VEGFR2, VE-Cadherin, Src and phospho-Src (Figure 2). Transient destabilization of VEGFR2/VE-Cadherin complexes was observed at 4 h reperfusion and was restored after 18 h reperfusion in angptl4LacZ/+ mice whereas VE-Cadherin remained dissociated from VEGFR2 in angptl4LacZ/LacZ mice at both time points (Figure 2A, upper panel). In addition, immunoblotting showed an increased phosphorylation of
Src kinase after 18 h of reperfusion in \( \text{angptl4}^{\text{LacZ/LacZ}} \) compared with \( \text{angptl4}^{\text{LacZ/+}} \) mice (Figure 2A, middle and bottom panels and 2B).

These results showed that a decrease in expression of VEGFR2 and VE-Cadherin combined with an increase in the phosphorylation of Src kinase downstream of VEGFR2 lead to dissociation of the VEGFR2/VE-Cadherin complex. Both events are responsible for massive disorganization of VE-cadherin in endothelial adherens junctions in \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice after ischemia-reperfusion.

**Infarct size, no-reflow and post-ischemic inflammation are increased in \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice**

We next hypothesized that alteration of vascular integrity in \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice might be translated to abnormal myocardial reperfusion and damage to heart tissue at 48 h reperfusion. Indeed, infarct size was increased in \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice compared with \( \text{angptl4}^{\text{LacZ/+}} \) mice (47±3% *versus* 36±3%, *p*<0.01) (Fig. 3A). In addition, no-reflow was more important in the \( \text{angptl4}^{\text{LacZ/LacZ}} \) group compared with \( \text{angptl4}^{\text{LacZ/+}} \) mice if expressed as a percentage of the area at risk (19±1% *versus* 11±2%, *p*<0.05) (Supplemental Table 2).

Necrosis, hemorrhage and edema were also quantified (score 1 to 3) on H&E-stained sections from infarcted hearts of both genotypes (Figures 3B and 3C). In accordance with increased infarct size, tissue necrosis was increased in \( \text{angptl4}^{\text{LacZ/LacZ}} \) compared with control mice (2.5±0.6 *versus* 1.2±0.2) (Figure 3D). Similarly, assessment of hemorrhage and edema revealed more severe tissue injury in \( \text{angptl4}^{\text{LacZ/LacZ}} \) mice (2.3±0.6 *versus* 1.1±0.2 and 2.3±0.2 *versus* 0.8±0.2, respectively, Figure 3D). The post-ischemic inflammatory response was also analyzed in both genotypes (Figures 3E to 3G). Macrophage density was significantly higher in
infarcted areas in \text{angptl4}_{LacZ/LacZ} \text{ versus } \text{angptl4}_{LacZ/+} \text{ mice, whereas no statistical difference was observed between the two groups in non-infarcted areas (Figure 3G).}

We then analyzed vascular density in the core infarct area and in the periphery using CD31 staining (Figures 3H and 3I). A similar diminished microcapillary density was quantified in both genotypes in the central infarcted areas as compared with the periphery. No difference was quantified between both genotypes in both areas (Figure 3J).

Transmission electron microscopy study was undertaken to assess tissue injury at the ultrastructural level. Analyses of reperfused infarcted areas did not show structural differences in cardiomyocytes between the two groups (see C1 to 4 in Supplemental Figures 3A and 3B). In contrast, large edematous areas with few inflammatory cells were observed in \text{angptl4}_{LacZ/LacZ} \text{ mice, whereas inflammatory cells had already invaded the edematous region in } \text{angptl4}_{LacZ/LacZ} \text{ mice (see E in Supplemental Figures 3C and 3D). Polynuclear neutrophils, macrophages (M\Phi), lymphocytes (L) and fibrinogen deposits (F) were observed only in } \text{angptl4}_{LacZ/LacZ} \text{ mice (Supplemental Figure 3D). Taken together, these data indicated increased vascular alterations that correlated with increased inflammatory infiltrates in } \text{angptl4}_{LacZ/LacZ} \text{ mice.}

In addition, hypoxic activation of \text{angptl4} mRNA has been reported in cardiomyocytes \text{in vitro}, probably mediated by hypoxia-inducible factor 1 (HIF1) \text{24}. Using \text{in situ} hybridization (ISH), we demonstrated \text{angptl4} mRNA expression \text{in vivo} in cardiomyocytes and in ECs in cardiac samples from patients who died from AMI (Supplemental Figure 4). As in humans, ISH carried out in mice subjected to ischemia-reperfusion showed that \text{angptl4} mRNA expression was induced in cardiomyocytes and in ECs (Supplemental Figure 5A and 5B). LacZ staining undertaken on H&E-stained and CD31-immunostained sections and on whole-mount from \text{angptl4}_{LacZ/LacZ} \text{ mice further confirmed expression in cardiomyocytes and ECs after ischemia-}
reperfusion injury (Supplemental Figures 5C to 5F, see arrowheads and arrows, respectively). We therefore tested the hypothesis that ANGPTL4 might affect cardiomyocyte survival in hypoxic conditions. Mouse cardiomyocytes from both genotypes were isolated and subjected to an in vitro survival assay. No difference in cardiomyocyte survival was observed in vitro between both groups in normoxia or hypoxia, suggesting that ANGPTL4 does not have a direct effect on cardiomyocytes (Figure 3K), thereby further suggesting it targets ECs.

Recombinant ANGPTL4 stabilizes the VEGFR2/VE-Cadherin complex in response to VEGF

We next investigated if rhANGPTL4 might rescue the more severe ischemia-reperfusion induced cardiac damage in angptl4\textsuperscript{LacZ/LacZ} mice. When injected in angptl4\textsuperscript{LacZ/LacZ} mice at 10 μg/kg body weight 5 min before ischemia-reperfusion, rhANGPTL4 reduced infarct size to 41±4% compared with 67±7% when mice were injected with vehicle (p<0.05, Figure 4A). Remarkably, the infarct size of rhANGPTL-injected angptl4\textsuperscript{LacZ/LacZ} mice was indistinguishable from that of control angptl4\textsuperscript{LacZ/+} mice (47±2%) injected with vehicle, showing that ANGPTL4 could rescue the knockout phenotype in mice.

We then sought to define in vitro the molecular pathways responsible for rhANGPTL4-induced cardioprotection by testing if rhANGPTL4 could confer protection of disassembly of the VEGFR2/VE-Cadherin complex. Confluent HUAECs were stimulated for 5 min with VEGF alone or with rhANGPTL4. Cell lysates were immunoprecipitated for VEGFR2 followed by immunoblotting for VEGFR2, VE-Cadherin, Src and phospho-Src. The pre-existing VEGFR2/VE-Cadherin observed in control conditions was rapidly disrupted within 5 min of VEGF stimulation. This complex was protected from dissociation in cells treated with both
VEGF and rhANGPTL4 (Figure 4B left and 4C). Src kinase and phospho-Src immunoblotting revealed that VEGF-mediated destabilization of the VEGFR2/VE-cadherin complex was correlated with an increased phosphorylation of Src downstream of VEGFR2 that was reduced in cells treated with both VEGF and rhANGPTL4 (Figure 4B right and 4C).

**Assessment of the therapeutic cardioprotective effects of rhANGPTL4 in a non-rodent species**

We next hypothesized that attenuation of vascular alterations by rhANGPTL4 may lead to the enhancement of endothelial barrier function, which ultimately could protect ECs from ischemia-induced reperfusion injury. Myocardial ischemia-reperfusion does not induce massive no-reflow in mice under these conditions, so we sought to analyze the therapeutic potential of ANGPTL4 in a non-rodent species. We chose an open-chest model of myocardial ischemia-reperfusion in rabbits because the no-reflow phenomenon has been well established in this model.

We undertook intravenous injection of 10 μg/kg rhANGPTL4 or vehicle, 5 min before ischemia-reperfusion. Infarct size (percentage area at risk) was 57±5% in the control group and 34±7% in the rhANGPTL4-treated group (p<0.01, Figure 5A). Then, the zone of no-reflow was studied. When expressed as a percentage of the area at risk, it was 41±2% in the control group and 19±6% in the rhANGPTL4-treated group (p<0.05, Figure 5B). More importantly, when expressed as a percentage of the infarct size, it was 73±4% in the control group and 55±7% in the rhANGPTL4-treated group (p<0.05, Figure 5C). Histological analyses showed that myocardial infarction comprised a core of necrosis and huge hemorrhage within interstitial spaces in the control group (Figures 5D and 5E). In the rhANGPTL4-treated group, the extent of
hemorrhage was decreased (5.7±2% versus 21.9±6.4%, expressed as a percentage of total heart section area, p<0.05) (Figure 5F).

Thus, these results showed that rhANGPTL4 induced preservation of vascular integrity that reduced infarct size, hemorrhage and no-reflow, thereby conferring cardioprotection.

**Discussion**

Upon AMI, HIF proteins, the major transcription factors involved in the regulation of responses to hypoxia, are rapidly activated and induce VEGF-A expression that participates to the angiogenic response. However, VEGF-A also causes vascular permeability and edema, resulting in extensive injury. We showed here that angptl4 mRNA, which was previously shown to be induced by hypoxia in ECs and in cardiomyocytes in vitro as well as in critical hindlimb ischemia and stroke, was also expressed in cardiac tissue from patients who died from AMI. In the present study, we provide evidence that ANGPTL4 mediates protection from post-ischemic tissue damage through preservation of the integrity of the vascular ECs barrier that limits no-reflow and the extent of AMI.

In pathological ischemic conditions, increased permeability, which is controlled by endothelial junction stability, is responsible for altered vascular integrity. VE-Cadherin, which constitutes the major component of the adherens junctions between ECs, is required in the post-natal vasculature to maintain the integrity and barrier function of the endothelium. Yang et al. showed that myocardial VE-Cadherin is significantly decreased in the ischemic myocardium, suggesting that microvascular integrity is damaged by ischemia-reperfusion. VE-Cadherin associates with VEGFR2 and regulates permeability. Indeed, systemic VEGF-A injection, thereby activating VEGFR2, induces dissociation of the VEGFR2/VE-cadherin complex.
complex \textsuperscript{38}. Here, we showed durable dissociation of VEGFR2/VE-cadherin complexes and altered VE-Cadherin distribution in \textit{angptl4\textsuperscript{LacZ/LacZ}} mice that caused disrupted adherens junctions and decreased EC barrier function after AMI. Gene and protein expression analyses revealed: (i) more prominent diminished VEGF and VE-cadherin levels, (ii) prolonged decrease in \textit{vegfr2} mRNA levels in \textit{angptl4} knockout mice subjected to AMI. In the heart, decreased levels VEGFR2/VE-Cadherin complexes in ECs in response to ischemia, might participate in junction disruptions and altered endothelial integrity after AMI in \textit{angptl4\textsuperscript{LacZ/LacZ}} mice. Src is an essential molecule required for promoting the disruption of EC contacts and paracellular permeability \textsuperscript{38}. We here provide evidence for enhanced Src kinase phosphorylation leading to more severe destabilization of the VEGFR2/VE-Cadherin complex in \textit{angptl4\textsuperscript{LacZ/LacZ}} mice subjected to AMI. Other members of the Angiopoietin family also have a role in the regulation of vascular permeability. Angiopoietin-1 phosphorylates Tie2 and phosphatidylinositol 3-kinase, inducing activation of the GTPase Rac1, which is needed to maintain cell–cell adhesion \textsuperscript{39} and which also activates mDia, resulting in sequestration of Src \textsuperscript{40}. Whether regulation of Src/mDia or Rac1 signaling pathways by ANGPTL4 affects intracellular VE-Cadherin distribution deserves further investigation.

Preservation of the microcirculatory network and, to a lesser extent, preservation of hemorrhage by rhANGPTL4 limited the extent of no-reflow. This phenomenon is the result of incompletely characterized anatomical changes of the coronary microcirculation \textsuperscript{3} in which ANGPTL4 might play a crucial role through its vasculoprotective effect. In addition, reperfused myocardial infarction is associated with cellular infiltration and the acute inflammatory response \textsuperscript{41}. A critical point in post-ischemic therapy is containment of the deleterious, persistent, and expanding inflammatory response \textsuperscript{42}. We showed here that altered vascular integrity in
angptl4<sup>LacZ/LacZ</sup> mice might suppress a point of control that participates in limiting the post-infarction inflammatory response and expansion of the infarcted area.

Our results showing disorganization of endothelial adherens junctions in angptl4<sup>LacZ/LacZ</sup> mice suggest that ANGPTL4 could promote the endothelial barrier function at multiple levels. We showed that endothelial barrier integrity was perturbed in angptl4<sup>lacZ/LacZ</sup> mice, and that rhANGPTL4 could restore some degree of integrity and protect mice and rabbits during ischemia-reperfusion. In addition, ANGPTL4 modulates the balance between circulating TG-rich lipoproteins, very-low-density lipoproteins (VLDLs) and chylomicrons (as well as their uptake) by inhibiting endothelial lipase and lipoprotein lipase anchored at the surface of ECs<sup>43, 44</sup>. In angptl4<sup>lacZ/LacZ</sup> mice, which display decreased circulating levels of TGs, loss of inhibition of lipoprotein lipase could lead to increased lipolysis products such as oxidized fatty acids which induce EC inflammation<sup>45</sup>. This could then participate in altered endothelial barrier integrity and therefore endothelial function<sup>46</sup>. Elucidation of the precise mechanisms linking ANGPTL4 activity, TG uptake and potential fatty acid-induced oxidative stress<sup>25</sup> needs to be definitively addressed. Nevertheless, markers of oxidative stress such as <i>fkbp5</i> and <i>gsta3</i> were not differentially regulated in angptl4<sup>lacZ/LacZ</sup> mice compared with control mice during ischemia-reperfusion (data not shown), showing that this is not likely to be the case. Alternatively, ANGPTL4-mediated dysregulation of VEGF-B control of endothelial uptake of fatty acids may also be involved<sup>48</sup>.

Finally, whereas cardiomyocytes are primarily recognized as the therapeutic target of myocardial ischemia, few studies have focused on the importance of heart vessels in this context. Clinical efforts are underway to block VEGF-A-mediated leak in patients after AMI<sup>49</sup> or stroke<sup>50</sup>. The present findings show that ANGPTL4 counteracts the increase in permeability observed
in reperfused AMI. This justifies the search for combined strategies that will have a significant impact on reducing tissue injury and improving the coronary microcirculation and the no-reflow phenomenon, thereby improving AMI therapy.

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**References:**


**Figure Legends:**

**Figure 1:** Leakage of Evans blue dye in the hearts of *angptl4*<sup>LacZ</sup>/<sup>+</sup> and *angptl4*<sup>LacZ/LacZ</sup> mice under basal conditions (n=3 for both groups) or if subjected to 45 min of ischemia and 4 h of reperfusion (n=3 for both groups) (A). Confocal microscopic images of VE-Cadherin immunoreactivities undertaken on hearts under basal conditions (B and C) or subjected to 45 min of ischemia and 4 h of reperfusion (D and E) from *angptl4*<sup>LacZ/+</sup> (left panel) and *angptl4*<sup>LacZ/LacZ</sup> mice (right panel). Arrows, arrowheads and open arrowheads show linear, thin and disrupted VE-Cadherin staining, respectively. Asterisks indicate extravasated FITC-beads. Scale bar=20 μm (B to D). Three dimensional-image rebuilt from confocal images showing extravasation of FITC-beads (green) from hearts subjected to ischemia-reperfusion and stained with anti-VE-Cadherin antibody (red) (F and G). Open circles represent individual values and closed circles represent mean±SEM. *P<0.05 indicates significantly different from respective controls.

**Figure 2:** VEGFR2 immunoprecipitation analyses were undertaken on extracts from left ventricles under basal conditions (CTL) or subjected to 45 min ischemia and 4 h or 18 h coronary artery reperfusion (CAR). The graphs show VE-Cadherin (n=2 for each group and condition), Src kinase (n=3-3, n=3-4, n=4-3) and phospho-Src (n=5-5, n=5-6, n=6-4) normalized to VEGFR2 in *angptl4*<sup>LacZ/+</sup> and *angptl4*<sup>LacZ/LacZ</sup> mice, respectively) (A). Src kinase and phospho-Src immunoblotting of *angptl4*<sup>LacZ/+</sup> (left panel) and *angptl4*<sup>LacZ/LacZ</sup> mice (right panel) (B). Data are expressed as a percentage of the control under basal conditions. Open circles represent
individual values and closed circles represent mean±SEM. The Friedman test (2-factor designed analysis), followed by Mann-Whitney U test we used for individual comparison at each time point. *P<0.05 indicates significantly different from respective controls.

**Figure 3:** Comparison of infarct size expressed as the percentage of area at risk between angptl4LacZ/+ and angptl4LacZ/LacZ mice subjected to 45 min of ischemia and 48 h of reperfusion (both n = 8). (A). Necrosis(n=4-4), hemorrhage (n=4-3) and edema (n=4-4) were then assessed in H&E-stained sections showing increased tissue damage in angptl4LacZ/+ and angptl4LacZ/LacZ mice, respectively) (B to D). Sections from infarcted heart were immunolabeled for inflammatory cells (E to G) and endothelial cells (H to J). Quantification of macrophages in control non-infarcted areas and in infarcted areas are shown in G (8 fields for both control and ischemia). Microvascular density was quantified in ischemic and necrotic areas (periphery, n=5-10 fields and core, n=4-4) (J). Scale bar=100 μm. Cardiomyocytes isolated from angptl4LacZ/+ and angptl4LacZ/LacZ mice (both n=4) subjected to a viability assay in normoxic or in hypoxic conditions (K). Open circles represent individual values and closed circles represent mean±SEM. *P<0.05 indicates significantly different from respective controls.

**Figure 4:** Infarct sizes (expressed as a percentage of the area at risk) in vehicle injected-angptl4LacZ/+, in vehicle injected-angptl4LacZ/LacZ, and in 10 μg/kg rhANGPTL4-injected angptl4LacZ/LacZ mice. (A). Western blot analyses were undertaken on HUAECs treated or not treated for 5 min with VEGF±ANGPTL4. Cell lysates were immunoprecipitated with an anti-VEGFR2 antibody. Graphs show VE-Cadherin normalized to VEGFR2 (n=3 for each condition) and the ratio between Src kinase and phospho-Src normalized to VEGFR2 (n=4 for each
condition). Data are expressed as a percentage of the control under basal conditions. Kruskall-Wallis tests followed by Mann-Whitney test were used for comparisons. Open circles represent individual values and closed circles represent mean±SEM. *P<0.05 indicates significantly different from respective controls. (B). VEGFR2, VE-Cadherin, Src kinase and phospho-Src immunoblotting (C).

**Figure 5:** Infarct sizes (expressed as a percentage of the area at risk) in control rabbits and in 10 μg/kg rhANGPTL4-injected rabbits (n=4 in both groups). Open circles represent individual infarct size and closed circles represent mean±SEM. *p<0.05 (A). Extent of the no-reflow zone expressed as a percentage of the area at risk (B) or expressed as a percentage of infarct size (C) in control and in 10 μg/kg rhANGPTL4-injected rabbits. Open circles represent individual no-reflow zones and closed circles represent mean±SEM. *p<0.05. H&E-stained sections in the control (D) and the rhANGPTL4-treated (E) group. Quantification of the surface of hemorrhage expressed as a percentage of the total section surface in both groups. Open circles represent individual quantification of the surface of hemorrhage and closed circles represent mean±SEM (F). Scale bar=1000 μm. The Friedman test (2-factor designed analysis), followed by Mann-Whitney U test we used for individual comparison at each time point. Open circles represent individual infarct size and closed circles represent mean±SEM. *P<0.05 indicates significantly different from respective controls.
Protection Against Myocardial Infarction and No-Reflow Through Preservation of Vascular Integrity by Angiopoietin-Like 4
Ariane Galaup, Elisa Gomez, Rachid Souktani, Mélanie Durand, Aurélie Cazes, Catherine Monnot, Jérémie Teillon, Sébastien Le Jan, Claire Bouleti, Gaëlle Briois, Josette Philippe, Sandrine Pons, Valérie Martin, Rana Assaly, Philippe Bonnin, Philippe Ratajczak, Anne Janin, Gavin Thurston, David M. Valenzuela, Andrew J. Murphy, George D. Yancopoulos, Renaud Tissier, Alain Berdeaux, Bijan Ghaleh and Stéphane Germain

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Supplemental material

Supplemental methods

Mice myocardial ischemia-reperfusion experiments: Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital. To assess infarct size and for immunohistochemistry (IHC), ultrastructural, biochemical or expression studies, male angptl4LacZ/+ and angptl4LacZ/LacZ mice were reperfused during either 4 h, 18 h or 48 h after ischemia. For rescue study mice randomly received either vehicle or human recombinant 55 kDa full-length ANGPTL4 (10 μg/kg i.v.) 5 min before the 45 min occlusion step followed by 48 h-reperfusion. The area at risk was identified by Evans blue staining at 48 h after ischemia, and the infarct area was identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The area at risk was identified as the non blue region and expressed as a percentage of the left ventricle weight. The infarcted area was identified as the TTC-negative zone and expressed as a percentage of the area at risk. To measure no-reflow, the chest was reopened and thioflavine S (4%; 1.5 mL/kg) was infused through the left atrium four hours after the onset of reperfusion. The hearts were then perfused retrogradely with Alcian blue (0.5%) and cut into slices. Slices were photographed using UV light to identify the region of no-reflow. The areas of infarct and risk zone were determined as defined above. Ultrastructural analyses were performed on a Hitachi H-9500 electron microscope.

Immunofluorescence study and confocal analysis on cryosections: Mice subjected to ischemia and 4 h of reperfusion were anesthetized with ketamine and xylazine injected intraperitoneally. FITC-beads (20 μL) were injected into the femoral vein as previously described. The chest was opened rapidly, and the vasculature was perfused for 2 min at a pressure of 120 mmHg with 1% paraformaldehyde. The heart was then placed into 1%
paraformaldehyde for 1 h at room temperature, rinsed with PBS and frozen for cryostat sectioning. ECs, pericytes and adherens junctions were identified with rat anti-CD31 (BD Pharmingen), rabbit anti-NG2 (Chemicon) and rat anti-VE-Cadherin (personal gift from E. Dejana, IFOM) antibodies, respectively. Confocal sections were imaged on a Leica SP5 microscope (Leica Microsystems GmbH) using a 63x (NA= 1.4) oil objective. An increment of 0.6 µm between each section was used. 3D reconstruction of the different structures was obtained using the LABELVOXEL and the SURFACEGEN modules in Amira 5.2.1 software (Visage Imaging GmbH).

**Immunoprecipitation and immunoblotting analyses**: Proteins were extracted on ice in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% DOC, 0.5% NP-40, 10% glycerol, 1 mM β-glycerophosphate, 1 mM NaF, 2.5mM Na pyrophosphate, 1 mM Na3VO4 and a cocktail of protease inhibitors (Calbiochem). Lysates were split for immunoprecipitation and for total extracts immunoblottsings. For immunoprecipitation, extracts were precleared for 60 min with protein A–agarose beads, incubated overnight with anti–VEGFR-2 (Cell signaling), and the immunocomplexes were collected on protein A–agarose beads for 3 h. Proteins were eluted by boiling for 10 min in reducing laemmLi sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting on nitrocellulose membrane. Anti-VEGFR2 (Cell signaling), anti-VE-Cadherin (Santa Cruz), anti-Src kinase family (Cell signaling), anti-phospho Src family Tyr- 416 (Cell signaling) antibodies were used. Signal was revealed by Attophos chemiluminescence (Promega) and band intensity was quantified by Quantity One 1-D Analysis Software (Biorad).

**Isolation of cardiomyocytes and viability assay**: Under anesthesia, the heart was removed from the chest and was cannulated. The heart was perfused for 4 min with tyrode buffer
(mM) NaCl 113; KCl 4.7; KH₂PO₄ 0.6; Na₂HPO₄ 0.6; HEPES 10; MgSO₄ 1.2; NaHCO₃ 12; KHCO₃ 10; taurine 30; phenol red 0.032; glucose 5.5; with pH adjusted to 7.46 with NaOH 1N) at constant pressure and 37°C. Perfusion was switched to an enzyme solution ([mM] NaCl 113; KCl 4.7; KH₂PO₄ 0.6; Na₂HPO₄ 0.6; HEPES 10; MgSO₄ 1.2; NaHCO₃ 12; KHCO₃ 10; taurine 30; phenol red 0.032; glucose 5.5; CaCl₂ 0.0125; with pH adjusted to 7.46 with NaOH 1N) containing 0.1 mg/mL liberase blendzyme IV, (Roche diagnostics) and 0.14 mg/mL trypsin (Sigma). When hearts became swollen and turn slightly pale, the atria and aorta were removed; the left ventricle were cut into small pieces and gently triturated. Cell suspension was transferred into a stopping buffer ([mM] NaCl 113; KCl 4.7; KH₂PO₄ 0.6; Na₂HPO₄ 0.6; HEPES 10; MgSO₄ 1.2; NaHCO₃ 12; KHCO₃ 10; taurine 30; phenol red 0.032; glucose 5.5; CaCl₂ 0.0125; calf serum 5%; with pH adjusted to 7.46 with NaOH 1N). Extracellular calcium was added incrementally up to 1.0 mM. All cells studied were rod-shaped, had clear cross-striations and lacked any visible vesicles on their surfaces.

**Rabbit experiments:** New Zealand rabbits (2.5-3.0 kg) were anesthetized using zolazepam, tiletamine and pentobarbital (all 20-30 mg/kg i.v.). The animals were intubated, mechanically ventilated and a left thoracotomy was performed. A suture was passed beneath a major branch of the left coronary artery through a short propylene tubing to form a snare. Rabbits then randomly received either vehicle or human recombinant 55 kDa full-length ANGPTL4 (10 µg/kg i.v.). Five minutes after, coronary artery occlusion (CAO) was induced during 30-min by pulling the snare through the tubing. Reperfusion was subsequently induced by releasing the snare. The chest was then closed in layers. Four hours after the onset of reperfusion, the chest was reopened and thioflavine S (4%; 1.5 mL/kg) was infused through the left atrium. Rabbits were then sacrificed using pentobarbital followed by potassium chloride. After excision, the hearts were perfused retrogradely with Alcian blue (0.5%) and cut into slices.
Slices were photographed using UV light to identify the region of no-reflow. The areas of infarct and risk zone were determined as in mice.

**Ultrasound analysis of cardiac parameters:** Mice were subjected to ultrasound measurements using an echocardiograph (Vivid 7, GE Medical Systems Ultrasound) equipped with a 12-MHz linear transducer.

**Real-time quantitative PCR analysis (RT-qPCR):** Mice subjected to ischemia and 4 h or 18 h reperfusion were anesthetized, perfused for 2 min at a pressure of 120 mmHg with PBS and dissected to remove left ventricle. Total RNA was isolated by extraction with TRIzol (Invitrogen). Reverse transcription, quantitative PCR (in triplicate) and analysis were performed as previously described 3. The following primers were used: mouse *ve-cadherin*, 5'-CACTGCACATCTACGGCTACG -3 and 5'- CAGTCGTTGAGGAAGTCATAATCG -3; mouse *vegfr2* gene was detected using pre-designed primer pairs (QuantiTect primer assays, QIAGEN). mRNA expression level was normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold changes were calculated using the comparative Ct method.

**In situ hybridization (ISH) and immunohistochemistry (IHC) analyses:** Paraffin blocks of human infective endocarditis, congenital heart disease and myocardial infarcts were obtained from the Pathology Department of Georges Pompidou European Hospital, Paris, France. The presence of infarcted areas was assessed on standard HE staining and adjacent slides were used for ISH and IHC analyses. To assess *angptl4* mRNA expression in mice, male *angptl4*\(^{lacZ/+}\) and *angptl4*\(^{LacZ/LacZ}\) mice were reperfused during either 48 h after 45 min
ischemia. ISH using human or mouse angptl4 probes, LacZ staining and IHC immunolabellings anti-CD45, -Mac3, and-CD31 were performed as previously described ⁴.
## Supplemental Tables

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Galaup *et al*, Table I
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Galaup et al., Table II
Galaup et al., Supplemental figure 1
Galaup et al, Supplemental figure 2
angptl4 $^{\text{LacZ/+/+}}$  

A  

\(C_1\)  
\(C_2\)  
\(C_3\)  
\(C_4\)  
\(V\)  

angptl4 $^{\text{LacZ/LacZ}}$  

B  

\(E\)  
\(V\)  
\(C_1\)  
\(C_2\)  

C  

\(C_1\)  
\(C_2\)  
\(E\)  
\(V\)  

D  

\(E\)  
\(M\Phi\)  
\(V\)  
\(F\)  
\(L\)  

E  

\(Pc\)  
\(V\)  

F  

\(Pc\)  
\(V\)  

Galaup et al, Supplemental figure 3
### B

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Galaup et al, Supplemental figure 4
Galaup et al, Supplemental figure 5
Supplemental Table Legends:

Supplemental Table 1: Measures of cardiac parameters assessed by ultrasound examinations performed on $angptl4^{lacZ/+}$ and $angptl4^{lacZ/LacZ}$ adult mice. Data are representative of 6 mice.

Supplemental Table 2: Extent of the no-reflow zone expressed as a percentage of the area at risk in $angptl4^{lacZ/+}$ mice compared to $angptl4^{lacZ/LacZ}$ mice subjected to ischemia and 48h reperfusion; *$p<0.05$ (B). Data are representative of 6 mice.

Supplemental Figures Legends:

Supplemental Figure 1: Confocal microscopic images of CD31 and NG2 immunoreactivities performed on frozen cardiac sections under basal conditions (A and B) or after 45 min ischemia-4 h reperfusion (C and D) in $angptl4^{lacZ/+}$ (left panel) and $angptl4^{lacZ/LacZ}$ adult mice (right panel). Scale bar=20 μm (A to D). Three dimensional-image rebuilt from confocal images showing extravasation of FITC-beads (green) from hearts subjected to ischemia-reperfusion stained with anti-CD31 (red) and anti-NG2 (blue) antibodies. Scale bar=2 μm (E and F). Data are representative of at least 3 experiments.

Supplemental Figure 2: Expression of vegfr2 mRNA (left), ve-cadherin mRNA (right) measured by RT-qPCR, in left ventricles isolated from $angptl4^{lacZ/+}$ and $angptl4^{lacZ/LacZ}$ mice in basal conditions (CTL) or subjected to 45 min ischemia and 4 h or 18 h reperfusion. RT-qPCR were performed in triplicate (A). Immunoblots of left ventricle of $angptl4^{lacZ/+}$ and $angptl4^{lacZ/LacZ}$ mice in basal conditions or subjected to 45 min ischemia and 4 h or 18 h reperfusion using VEGFR2, VE-Cadherin, and actin antibodies (B). Bar graphs show
VEGFR2 and VE-Cadherin protein expression levels normalized to actin (left and right, respectively). Bar graphs represent mean± SEM of 3 experiments. Differences were considered significant with *, p<0.05.

**Supplemental Figure 3:** Transmission electron micrographs of infarcted hearts from *angptl4*<sup>LacZ/+</sup> and *angptl4*<sup>LacZ/LacZ</sup> mice (left and right panels, respectively). Cardiomyocytes are marked by « C<sub>1 to 4</sub> », edema by « E », macrophages by « Mϕ », lymphocytes by « L », fibrinogen deposits by « F », vessels by « V» and pericytes by « Pc ». Cardiomyocytes in infarcted areas are shown in A and B. At higher magnification, analysis of edema (C&D) revealed the infiltrating macrophages and lymphocytes in *angptl4*<sup>LacZ/LacZ</sup> mice. Pericytes are closely apposed to endothelial cells in *angptl4*<sup>LacZ/+</sup> vessels (E) whereas basement membrane from *angptl4*<sup>LacZ/LacZ</sup> vessels are edematous (arrow in F). Data in A–F are representative of at least 4 mice. Scale bar=5 μm in A, B and D. Scale bar=2 μm in C, E and F.

**Supplemental Figure 4:** *In situ* hybridization analysis of *angptl4* mRNA levels in infective endocarditis (1 and 2), congenital heart disease (3 to 5) and AMI (6 to 10) in humans (A and B). At higher magnification, *angptl4* mRNA after AMI was identified in both endothelial cells (top) and cardiomyocytes (bottom) around altered necrotic ischemic areas (N) (C). Scale bar=80 μm in A; 40 μm in C (upper panel) and 20 μm in C (upper panel).

**Supplemental Figure 5:** Histological analyzes performed in wild-type mice subjected to 45 min ischemia-48h reperfusion. H&E-stained sections showing infarcted area around areas of necrosis shown with arrowheads (A). *In situ* hybridization analysis of *angptl4* mRNA levels in adjacent slides showing murine *angptl4* mRNA expression in cardiomyocytes and endothelial cells (arrows) and showing necrosis areas (arrowheads) (B). β-galactosidase
staining recapitulating the *bona fide* expression pattern of the *angptl4* gene in the ischemic area of the heart of *angptl4*\textsuperscript{LacZ/LacZ} mice subjected to 45 min ischemia/48 h reperfusion (C to F). β-galactosidase staining in cardiomyocytes, arrowheads (C) and ECs, arrows (D and E). Double-labeling was performed using an anti-CD31 antibody in order to confirm endothelial cells identity (E). Whole-mount β-galactosidase staining showing staining downstream the coronary artery ligation (asterisk), atria were removed prior to β-galactosidase staining (F). Data are representative of at 4 mice. Scale bar=100 μm in A and B, 50 μm in C to E, 1000 μm in F.
Supplemental References:


